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Genotyping *Mycoplasma hyorhinis* by multi-locus sequence typing and multiple-locus variable-number tandem-repeat analysis

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Highlights

- Development of high resolution and robust MLVA and MLST for genotyping *M. hyorhinis*
- 40 *M. hyorhinis* strains isolated from Central Europe were genotyped
- High variability of *M. hyorhinis* strains was detected
- Combined use of MLST and MLVA for reliable phylogenetic studies is recommended

Abstract

Mycoplasma hyorhinis is a swine pathogen bacterium, which causes significant economic losses. The infection spreads through direct contact between the animals. Powerful genotyping methods like PCR based multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA) are necessary to monitor the infections and to conduct epidemiological investigations; hence supporting the control of the disease. The aims of the present study were to examine *M. hyorhinis* isolates originating mainly from Hungary with MLST and MLVA developed in the study, and to compare the results of the two typing methods.

To characterize 39 *M. hyorhinis* isolates and the type strain (NCTC 10130), six house-keeping genes were selected for MLST and six tandem-repeat regions were chosen for MLVA. We were able to differentiate 31 sequence types and 37 genotypes within the 40 analyzed isolates by the MLST and the MLVA, respectively. With the combination of the two newly developed assays all examined isolates were distinguished with the exception of the ones originating from the same animal. The developed MLST assay provided a robust and high resolution phylogenetic tree, while the MLVA system is suitable for the differentiation of closely related isolates from the same farm, hence the assay is appropriate for epidemiologic studies.

Keywords

Differentiation; genotyping; MLST; MLVA; *Mycoplasma hyorhinis*; swine

Introduction

Mycoplasma hyorhinis, first described by Switzer in 1955 (Switzer, 1955), is a swine pathogen bacterium distributed worldwide with an estimated occurrence around 50-70% in the herds (Hansen et al., 2010). As *M. hyorhinis* is a facultative pathogen bacterium, the manifestation of clinical signs are triggered by stress or presence of co-infections. Systemic infections occur mainly in 3-10 weeks old piglets, with typical symptoms like arthritis, polyserositis and less often otitis and conjunctivitis (Rovira et al., 2010). The pathogen is transmitted by direct contact, the piglets first make contact with the bacteria through gilts and sows, and then *M. hyorhinis* spreads between pigs when they are grouped together post-weaning. In the past years, *M. hyorhinis* was detected more often in piglets showing these signs and became one of the main concerns of swine veterinarians dealing with post-weaning infections (Clavijo et al., 2017).

A vaccine against *M. hyorhinis* infections is commercially available (Ingelvac MycoMAX™, Boehringer Ingelheim Animal Health USA Inc., Duluth, USA); however, up to date its use is only authorized in the United States. Thus, beside vaccination, protection against the infection still relies upon prevention, antibiotic treatment and better understanding of the pathogen. Well standardized and comparable genotyping methods, like the PCR based multi-locus sequence typing (MLST) and multiple-locus variable-number tandem-repeat analysis (MLVA) are powerful tools to determine the source of infection and describe phylogenetic relationships among the strains.

MLST is suitable to detect mid-term evolutionary distances and to determine relationships between the strains based on point mutations in house-keeping genes (Maiden, 2006). In phylogenetic analyses, bootstrap method is used to assess confidence. Based on the work of Hillis and Bull (Hillis and Bull, 1993) internal branches (nodes) with bootstrap proportions

above 70% represent true clades thus we consider these nodes well supported. The robustness of the phylogenetic tree is determined based on the bootstrap values of the nodes. An MLST system was published before for genotyping *M. hyorhinis* isolates (Tocqueville et al., 2014) and it was optimized by Trüeb et al. (2016). Currently, the MLST profiles of 133 *M. hyorhinis* strains are available at the PubMLST database (https://pubmlst.org/mhyorhinis/), which is established on the published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016). The typing of these strains resulted in discriminating 104 sequence types (STs), but according to the bootstrap values of the resulted phylogenetic tree (Maximum Likelihood algorithm, Tamura-3 model with MegaX software; Kumar et al., 2018) the robustness of the tree is low. In the last year a modified MLST-s (surface) system was also published, which uses two house-keeping genes (*pdhB, ung*) and two surface-encoding protein genes (*p95, mtlD*) to increase the discriminatory power of the test (Clavijo et al., 2019).

MLVA discriminates strains based on the repeat number of tandem-repeat (TR) units in the genome. These regions usually harbor several mutations and have higher mutation rates than the house-keeping genes, therefore can be used for the analysis of short-term evolutionary changes and applicable in epidemiologic studies (Nadon et al., 2013). An MLVA system for *M. hyorhinis* was published before, where two variable-number tandem-repeat regions in hypothetical proteins (Mhr_0152, Mhr_0298) were analyzed (Dos Santos et al., 2015).

The aims of the present study were to characterize mainly Hungarian *M. hyorhinis* isolates with already published and novel MLST and MLVA systems and compare the methods by examining a number of recent clinical isolates.

Materials and methods

Samples

A total of 46 *M. hyorhinis* strains were used in the study: 38 field isolates originating from Hungary, one Slovakian isolate, the *M. hyorhinis* type strain (NCTC 10130; GenBank Accession number: LS991950.1) as control strain, and six available whole genome sequences [HUB-1 (GenBank: CP002170.1), GDL-1 (GenBank: CP003231.1), SK76 (GenBank: CP003914.1), DBS1050 (GenBank: CP006849.1), MCLD (GenBank: CP002669.1) and MDBK-IPV (GenBank: CP016817.1)] (Supplementary table 1). Different kinds of clinical samples were collected such as tissue of lung, tonsil or cerebra, meninx or serous membrane, exudate from pericardium or thoracic cavity, synovial fluid from joints or bursa and nasal swabs, between 2014 and 2019. According to the ethics committee of the Institute for Veterinary Medical Research, Centre for Agricultural Research ethical approval was not required for the study as the samples were taken either during routine diagnostic examinations with the consent of the owners or in slaughterhouses. The isolates were cultured as described before (Bekő et al., 2019a). DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturers' instructions for Gram-negative bacteria. All isolates were identified by polymerase chain reaction (PCR) targeting the *p37* gene of *M. hyorhinis* (Caron et al., 2000; Assunção et al., 2005). The presence of other *Mycoplasma* species was excluded by a universal *Mycoplasma* PCR system targeting the 16S/23S rRNA intergenic spacer region in Mollicutes (Lauerman et al., 1995) followed by sequencing on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems, Foster City, USA), sequence analysis and BLASTN search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Whole genome sequencing of 37 field isolates (all except MycSu32i and MycSu32s) was performed on NextSeq 500 Illumina (Illumina Inc., San Diego, USA) next generation sequencing platform with NextSeq 500/550 High Output Kit v2.5 reagent kit (Illumina Inc.), resulting in 150 bp long single reads. The reads were quality checked with FastQC software version 0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Short reads were

mapped to and annotated according to *M. hyorhinis* type strain (NCTC 10130) by Geneious Prime software version 2019.2.1. (Kearse et al., 2012).

MLST assay

House-keeping genes suitable for MLST were selected based on the whole genome sequences of the clinical isolates and other publicly available *M. hyorhinis* whole genome sequences (*M. hyorhinis* type strain (NCTC 10130), HUB-1, GDL-1, SK76, DBS1050, MCLD and MDBK-IPV). At first, 17 genes were selected based on MLST alleles described in *Mycoplasma* sp. before (Manso-Silván et al., 2012; Dijkman et al., 2016; Ghanem and El-Gazzar, 2016; Bekő et al., 2019b) including the ones from the published *M. hyorhinis* MLST (Tocqueville et al., 2014; Trüeb et al., 2016). Genes were selected based on genome position (even distribution) and variability.

The primers flanking these regions were designed based on the whole genome sequences of the present field isolates and the *M. hyorhinis* type strain (NCTC 10130). Melting temperatures and suitability were calculated using the NetPrimer software (Premier Biosoft International, Palo Alto, CA, USA) and the specificity of the primers were checked with BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences and fragment positions are listed in Table 1. Each fragment was amplified individually and their sequences were determined on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The PCR mixtures contained 5 µl 5× Green GoTaq Flexi buffer (Promega Inc., Madison, WI, USA), 2.5 µl $MgCl_2$ (25 mM; Promega), 0.5 µl dNTP (10 mM; Qiagen), 2 µl of each primer (10 pmol/µl), 0.25 µl GoTaq Polymerase (5U/µl, Promega) and 2 µl DNA template with a total volume of 25 µl. The PCR was performed on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). Thermocycling parameters were 95 °C for 3 minutes followed by 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for

45 seconds. Final elongation step was performed at 72 °C for 7 minutes. For the diversity analysis the sequences of the gene fragments were concatenated (3,560 bp total). The phylogenetic analysis was performed including all 39 clinical isolates (Supplementary table 1), the *M. hyorhinis* type strain (NCTC 10130) and six strains from GenBank (HUB-1, GDL-1, SK76, DBS1050, MCLD and MDBK-IPV) with Maximum Likelihood method, Hasegawa-Kishino-Yano model in the MegaX software (Kumar et al., 2018).

The MLST analysis of 44 strains [37 field isolates used in this study (omitting MycSu32i and MycSu32s), the six available *M. hyorhinis* whole genomes (HUB-1, GDL-1, SK76, DBS1050, MCLD and MDBK-IPV) and the *M. hyorhinis* type strain (NCTC 10130)] was performed according to the previously published MLST (Tocqueville et al., 2014; Trüeb et al., 2016) also, and MLST profiles were compared with sequences from the PubMLST database (https://pubmlst.org/mhyorhinis/). Gene fragments from *dnaA*, *rpoB*, *gyrB*, *gltX*, *adk* and *gmk* were amplified as described previously by Trüeb et al. (2016) and the PCR products were sequenced on an ABI Prism 3100 automated DNA sequencer (Applied Biosystems). The phylogenetic analysis was performed with Maximum Likelihood method, Tamura-3 model in the MegaX software (Kumar et al., 2018).

Based on the same 44 strains [37 field isolates used in this study (omitting MycSu32i and MycSu32s), the six available *M. hyorhinis* whole genomes (HUB-1, GDL-1, SK76, DBS1050, MCLD and MDBK-IPV) and the *M. hyorhinis* type strain (NCTC 10130)] comparison with the MLST-s system (Clavijo et al., 2019) was also performed and the MLST-s profiles were compared with the strains from the study of Clavijo et al. (2019). Gene fragments from *phdB*, *p95*, *mtlD* and *ung* were analyzed from the whole genome sequences. The phylogenetic analysis was performed with Maximum Likelihood method, Tamura-3 model in the MegaX software (Kumar et al., 2018).

MLVA assay

Variable-number of tandem-repeat (VNTR) regions were identified in the *M. hyorhinis* type strain (NCTC 10130) genome, and in six other available *M. hyorhinis* (HUB-1, GDL-1, SK76, DBS1050, MCLD, MDBK-IPV) genomes using the Tandem Repeat Finder program (Benson, 1999). At first, 23 loci were selected on the basis of period size (at least 12 bp) and the percent of insertions/deletions (0%). Primers were designed based on the *M. hyorhinis* type strain (NCTC 10130) and the six available whole genomes (HUB-1, GDL-1, SK76, DBS1050, MCLD, MDBK-IPV), melting temperatures and suitability were calculated using the NetPrimer software (Premier Biosoft International) and the specificity of the primers were checked with BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primer sequences and VNTR regions are listed in Table 2. The developed MLVA system was applied to characterize 39 *M. hyorhinis* field isolates and the type strain (NCTC 10130). The PCR mixtures contained 2.5 µl AmpliTaq™ Gold 10× PCR buffer (Applied Biosystems), 2.5 µl $MgCl_2$ (25 mM; Applied Biosystems), 1 µl dNTP (10 mM; Qiagen), 1 µl of each primer (10 pmol/µl), 0.2 µl AmpliTaq™ Gold Polymerase (5U/µl, Applied Biosystems) and 2 µl DNA template with a total volume of 25 µl. The PCR was performed on Bio-Rad C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories). Thermocycling parameters were 95 °C for 5 minutes followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds. Final elongation step was performed at 72 °C for 5 minutes. After amplification, 5-10 µl of each reaction mixture were submitted to electrophoresis. Electrophoresis of amplicons above 200 bp length was carried out in 2% (SeaKem® LE Agarose, Lonza Inc., Rockland, ME, USA) agarose gel with a 100-bp DNA ladder (GeneRuler 100 bp Plus, Thermo Fisher Scientific, Waltham, MA, USA), while amplicons below 200 bp were detected in 3% (MetaPhor Agarose, Lonza) agarose gel using a 20-bp DNA ladder (O'RangeRuler 20 bp, Thermo Fisher Scientific) as a molecular weight

marker. The amplified PCR products were visualized with ECO Safe Nucleic Acid Staining Solution (Pacific Image Electronics Inc., Torrance, CA, USA) under UV light.

Stained gels were photographically documented (Kodak Inc., Rochester, NY, USA) and band sizes were estimated with the Kodak MI SE software package (Kodak). The estimated band sizes were converted to number of repeat units based on the formula in Table 3. The clustering analysis was performed with Neighbor-Joining method based on pairwise distances in the MegaX software (Kumar et al., 2018).

Sensitivity and specificity of the assays

In order to test the sensitivity of the developed assays, tenfold dilutions of the type strain (NCTC 10130) were used in the range of 10^8-10^0 copy number/µl. Template copy number was calculated with the help of an online tool (Staroscik, 2004) by measuring the concentration of DNA of pure *M. hyorhinis* culture by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific). The lowest DNA concentrations detectable with agarose gel electrophoresis were considered the detection limits of the methods. The specificity was tested by including *M. hyopneumoniae*, *M. hyosynoviae* and *M. flocculare* in the analyses.

Comparison of the methods

The discriminatory power of the MLST systems were compared based on the Simpson's index of diversity (ID) and the variable nucleotides based on parsimony-informative sites. The Simpson's ID was determined using the online tool ComparingPartitions (http://www.comparingpartitions.info/). The variable nucleotides based on parsimony-informative sites were determined for the genes of the MLST systems individually and as concatenated sequences in MegaX software (Kumar et al., 2018). Phylogenetic trees based on the MLST profiles of the 44 isolates used in this study and determined by the three MLST based

systems were also compared. The diversity of the MLVA system described here was determined by calculating the Simpson's ID by the online tool ComparingPartitions.

In order to define the proportion of congruence between the MLST described here, the previously published MLST (Tocqueville et al., 2014; Trüeb et al., 2016), the MLST-s system (Clavijo et al., 2019) and the MLVA described here, the adjusted Rand coefficients were calculated by the online tool ComparingPartitions.

Results

Results of the M. hyorhinis MLST assay

After preliminary sequence analysis of 17 chromosomal genes of *M. hyorhinis* six genes were selected for development of an MLST assay based on the diversity among the clinical isolates, the type strain (NCTC 10130) and other available strains (HUB-1, GDL-1, SK76, DBS1050, MCLD, MDBK-IPV). For this purpose, the leader peptidase A (*lepA*), the DNA-directed RNA polymerase subunit beta (*rpoB*), the DNA-directed RNA polymerase subunit beta' (*rpoC*), the glutamyl-tRNA synthetase (*gltX*), the UvrABC system protein A (*uvrA*) and the valine-tRNA ligase (*valS*) genes were selected. In *rpoB* and *gltX* genes the amplified fragments (genome positions of *rpoB*: 71,638-72,295; *gltX*: 417,808-418,217; nucleotide numbering is according to *M. hyorhinis* type strain, NCTC 10130, GenBank: LS991950.1) differed from those targeted earlier by Tocqueville and co-workers (2014) in their MLST assay (*rpoB*: 71,195-71,690; *gltX*: 418,819-419,188). Sequences of the gene fragments from the MLST scheme described here are available in the GeneBank database (*lepA*: MT090880-MT090918, *rpoB*: MT090841-MT090879, *rpoC*: MT090802-MT090840, *gltX*: MT090919-MT090957, *uvrA*: MT090763-MT090801, *valS*: MT106126-MT106164). The sensitivity of the

PCR assays was between 10^2 and 10^5 copies/reaction (Table 1) and we detected cross-reaction with *M. hyopneumoniae* in case of the *valS* gene PCR.

From the concatenated sequences (3,560 bp total) a phylogenetic tree was made with Maximum Likelihood algorithm, Hasegawa-Kishino-Yano model in MegaX software (Figure 1). The analyzed 46 *M. hyorhinis* strains represented 37 sequence types (ST). The allele types by genes and STs for the concatenated sequences are listed in Supplementary table 1. The Hungarian isolates formed several subclades and branches. Distinct levels of relations were observed among isolates originating from the same farm. Eleven isolates from the same farm (Bácsalmás), isolated between 2014-2019, were examined. Most isolates from Bácsalmás belonged to a separate subclade representing two STs (ST2 and ST3), but the two latest isolates from herd A (MycSu141, MycSu152) formed a different branch with ST4 and ST5. The isolates in ST2 and ST3 differ only in one nucleotide in the *rpoC* gene fragment, while four single nucleotide polymorphisms (SNPs) were detected between ST4 and ST5 in the *rpoB* and *uvrA* gene fragments. There were nine nucleotides that differed between ST3 and ST4 in the *lepA*, *rpoB*, *rpoC*, *uvrA* and *valS* gene fragments, and five SNPs were identified between ST3 and ST5 in the *lepA*, *rpoC* and *valS* gene fragments. From Hajdúszoboszló three isolates were examined, collected between 2014-2016. Two isolates (MycSu24, MycSu27) represented the same ST (ST22) and the third one was labelled as ST30. Eight SNPs in four house-keeping genes (*lepA*, *gltX*, *rpoB* and *rpoC*) were detected between ST22 and ST30. Two isolates from Jánoshalma (MycSu86, MycSu103) was also examined, collected in 2016 and 2017. These two isolates represented two different STs, ST13 and ST8 respectively and SNPs (12 in total) in every studied gene fragments were found. No further correlations were identified between integration, year of isolation, sample type and ST. The three isolates originating from the same animal (MycSu32: synovial fluid (i), tonsil (t), serosa (s); Figure 1) shared the same ST (ST3).

The isolates examined in this study showed high diversity with the previously published MLST scheme (Tocqueville et al., 2014; Trüeb et al., 2016). A number of novel allele types were identified; in particular, one new allele type on genes *rpoB, gyrB* and *gmk,* three new allele types on gene *gltX* and four new allele types on gene *adk.* Collectively, our isolates represented 25 novel STs (Supplementary figure 1; Supplementary table 1). The new allele types, STs and the isolate data are available in the PubMLST database (https://pubmlst.org/mhyorhinis/).

The isolates examined in this study also showed high diversity with the MLST-s system (Clavijo et al., 2019). New allele types were identified; namely five new allele types on gene *phdB*, three new allele types on gene *p95* and 14 new allele types on gene *mtlD*. Collectively, our isolates represented 28 novel STs (Supplementary table 1, Supplementary Figure 1). Sequences of the gene fragments from the MLST-s scheme are available in the GenBank database (*mtlD*: MT569036 - MT569072; *p95*: MT568999 - MT569035; *phdB*: MT569073 - MT569109; *ung*: MT569110 - MT569146).

Results of the M. hyorhinis MLVA assay

A total of 75 TRs were found in the *M. hyorhinis* type strain's whole genome sequence. These were narrowed down to 23 amplified TRs based on length (>12 nucleotides) and lacking insertions or deletions in the repeat region. After preliminary amplifications, where some markers turned out to be monomorphic or presented multiple bands, six alleles (Mhr205, Mhr396, Mhr438, Mhr441, Mhr442, and Mhr444) were selected.

From the finally selected six markers three alleles (Mhr438, Mhr441, Mhr444) are highly diverse: the number of polymorphisms in these alleles ranged between 14 and 16. The other three alleles (Mhr205, Mhr396, Mhr442) were less diverse, there were three different number of repeat units in these alleles in the examined isolates. The repeat numbers for each TR and

the MLVA genotypes are listed in Supplementary table 1. The sensitivity of the MLVA PCRs was 10^3 copies/reaction and showed no cross-reaction with the tested *Mycoplasma* species.

The 40 *M. hyorhinis* isolates represented 37 genotypes (GT) based on the MLVA profiles, with Simspon's ID of 0.995 (CI 95% 0.986-1.000). By combining the MLST and MLVA methods all *M. hyorhinis* isolates were discriminated except for the isolates from different tissue samples of the same animal (MycSu32i, MycSu32s, MycSu32t; Figure 1). With the MLVA method, no correlation was found between sample source or integration and GT.

Comparison of the methods

The MLST described here has higher resolution (37 STs) than the previously described MLST based assays (31 STs both) (Tocqueville et al., 2014; Trüeb et al., 2016; Clavijo et al., 2019; Supplementary table 1, Supplementary table 2). On the phylogenetic tree based on the new MLST scheme five bootstrap values over 70% were found (Figure 1) while analyzing the same 44 isolates no bootstrap value over 70% was found with the previously published system (Tocqueville et al., 2014; Trüeb et al., 2016; Supplementary Figure 2). The MLST-s system (Clavijo et al., 2019) showed the highest number of bootstrap values (n=12) analyzing the same sample set (Supplementary figure 2). The diversity indices are summarized in Supplementary table 2.

To compare the results of the phylogenetic analyses isolates that were grouped together or originated from the same farm were examined with the MLST based systems. On the phylogenetic tree based on the published MLST scheme (Tocqueville et al., 2014; Trüeb et al., 2016) isolates from Bácsalmás represented four STs, similarly to the results of the presented MLST scheme. The two latest isolates from herd A represented ST114 (MycSu141) and ST115 (MycSu152), while isolates from the previous years formed ST105 (MycSu23, MycSu29, MycSu101, MycSu128) and ST107 (MycSu32t, MycSu127, MycSu150). ST105 and ST107

differ only in one nucleotide on the *gmk* gene, while two SNPs were detected between ST114 and ST115 on genes *dnaA* and *rpoB*. Nevertheless, the STs among the isolates from Bácsalmás determined by the here described system corresponded better with the available metadata (separating the isolates according to their year of isolation). On the phylogenetic tree based on the MLST-s system (Clavijo et al., 2019) the isolates from Bácsalmás were grouped together (ST49) except for the two latest isolates ST14 (MycSu141) and ST41 (MycSu152). According to the examined house-keeping genes no differences were found between the isolates from this farm, but four nucleotides differed on surface-encoding protein genes *mtlD* and *p95* between ST49 and ST14, while ST14 and ST41 differed in one SNP on gene *p95*.

Similarly to the here described MLST the three isolates from Hajdúszoboszló were grouped into two STs (ST40 and ST88) based on one SNP on gene *dnaA* with the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016). However, there were isolates from other farms that were grouped with the isolates from Hajdúszoboszló, MycSu55 for ST40 and MycSu90 for ST88. With the MLST-s (Clavijo et al., 2019) system the isolates from Hajdúszoboszló showed three different STs (ST52, ST53, ST64), but MycSu24 and MycSu27 differed only on the surface-encoding protein gene *mtlD*, while MycSu76 differed from these isolates in three nucleotides on the house-keeping gene *phdB.*

The two isolates from Jánoshalma showed similar results with all MLST systems, the two isolate represented ST124 and ST127 and differed in six SNPs on genes *gyrB*, *adk*, *dnaA* and *rpoB* with the previously published MLST (Tocqueville et al., 2014; Trüeb et al., 2016). While with the MLST-s (Clavijo et al., 2019) system the main differences were found on gene *mtlD* (30 SNPs), one nucleotide difference was found on gene *p95* and one SNP was detected on a house-keeping gene *phdB.*

With the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016) two isolates from different years and different farms MycSu63, MycSu106 were grouped

together in ST13, while they showed distinct profiles with the MLST-s system (Clavijo et al., 2019) and with the here described MLST. Two isolates from the same year but different farms (MycSu56, MycSu92) were also turned out to be similar and formed ST108 with the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016), but represented different STs with the MLST-s (Clavijo et al., 2019) system and differed with the here described system also.

Other isolates were grouped together with the MLST-s system but differed based on the other two MLST assays. MycSu64 and MycSu65 shared ST40 (MLST-s), while these two isolates were located on the same branch but differed in five nucleotides on genes *rpoB* and *gltX* with the here described MLST system, and were located on different branches of the phylogenetic tree based on the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016) differing in six nucleotides total on genes *gyrB*, *gltX*, *gmk* and *rpoB*. MycSu56, MycSu57 and MycSu58 were grouped together in ST54 (MLST-s) and represented different STs (ST26, ST25, ST27 respectively), but located on the same branch with the MLST described here. MycSu56 differs from MycSu57 in two nucleotides on genes *rpoB* and *gltX*, and differs from MycSu58 in 2 SNPs on *gltX*. These isolates also represented three different STs with the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016), MycSu56 differs from MycSu57 in one nucleotide on gene *gmk*, and differs from MycSu58 in four SNPs on genes *gyrB*, *dnaA* and *gltX*. Two other isolates MycSu59 and MycSu60 were grouped together with the MLST-s system (ST67; Clavijo et al., 2019) but located on different branches and differs in 14 SNPs on genes *lepA*, *rpoB*, *gltX*, *uvrA* and *valS* with the here described MLST system. Similarly, four SNPs were detected between the two isolates on genes *gyrB*, *adk* and *gltX* with the previously published MLST system (Tocqueville et al., 2014; Trüeb et al., 2016).

Interestingly, the isolates MycSu29 and MycSu101 from Bácsalmás, which showed identical MLVA GT and belonged to the same MLST STs based on the previously developed MLST (Tocqueville et al., 2014; Trüeb et al., 2016) and MLST-s (Clavijo et al., 2019) systems were differentiated by the developed MLST system, in accordance with the isolates epidemiologic background.

The adjusted Rand coefficients defining the proportion of congruence between the MLST systems were similar. Compared to the MLVA system, all MLST systems showed low congruence (Supplementary table 2).

Discussion

M. hyorhinis can cause significant economic losses in the swine industry due to reduced weight gain and increased treatment costs (Scheiber and Thacker, 2012). Therefore, better understanding of the bacteria with efficient genotyping tools is key to control and monitor the infections and to carry out epidemiological investigations. Here we described new, high resolution MLST and MLVA systems (Simpson's ID 0.985 and 0.995) to genotype *M. hyorhinis* isolates and to reveal the phylogenetic relations and epidemiological connections. Limitations of the study were that all isolates originated from routine diagnostic samplings or from slaughterhouses from mainly Hungary and a short period of time (2014-2019); thus complex epidemiologic analysis could not be carried out. However, with the help of the developed assays high diversity was detected (Supplementary Figure 1), similarly to the previously published *M. hyorhinis* genotyping studies (Tocqueville et al., 2014; Trüeb et al., 2016; Clavijo et al., 2019) and to a previous study with Central-European *M. hyopneumoniae* isolates (Felde et al., 2018). Although general conclusions can not be determined due to the

sampling frame of the study, several correlations were found among the analyzed clinical isolates and their genetic characteristics.

With the MLST system we were able to differentiate isolates from different farms, but not within farms which corresponds with the resolution of the assay (Maiden, 2006). From Bácsalmás (the herd with the highest isolate number) the isolates are separated by the year of isolation (ST2: isolates from 2014-2015, ST3: isolates from 2016-2019), so the difference can be due to an evolutional event, which is supported by the sole SNP in the *rpoC* gene fragment. The two latest isolates from herd A (MycSu141, MycSu152) differ greatly from the other isolates from Bácsalmás and from each other, which indicates that new strains were introduced later to the herd. Introduction of new strains was also detected in case of Hajdúszoboszló (ST22 and ST30) and Jánoshalma (ST8 and ST13), which was confirmed by the previously published MLST and MLST-s systems also (Tocqueville et al., 2014;Trüeb et al., 2016; Clavijo et al., 2019).

The two latest isolates from Bácsalmás (MycSu141 and MycSu152), isolated only six months apart, differ greater form each other than isolates from different years from the same farm. The detected isolates may represent examples for the possibility of the presence of more than one strain at the same farm at the same time; a finding detected earlier only in case of *M. hyopneumoniae* (Vranckx et al., 2011). The isolate from Beremend shared ST2 with two isolates from Bácsalmás (MycSu23, MycSu29), which can be explained by that the two farms, from where the isolates originated, belong to the same integration. However, we found no further evidence for correlation between ST and integration.

The novel and the previously published (Tocqueville et al., 2014; Trüeb et al., 2016; Clavijo et al., 2019) MLST schemes were compared based on the results of the phylogenetic analysis of the same 44 strains (Supplementary table 1). For the new MLST scheme the six most diverse gene fragments were selected from the originally analyzed 17 house-keeping genes, which

helped to increase the number of parsimony-informative sites from 0.65 parsim% (Tocqueville et al., 2014; Trüeb et al., 2016) to 1.12 parsim% (MLST scheme described here). However, the MLST-s system (Clavijo et al., 2019) has a higher number of parsimony-informative sites (parsim% 8.12), due to the use of highly variable surface protein coding genes. Nevertheless, the MLST assay described here showed higher resolution (Simpson's ID 0.985) than the previously published MLST system (Simpson's ID 0.975; Tocqueville et al., 2014; Trüeb et al., 2016) and even than the MLST-s system (Simpson's ID 0.962; Clavijo et al., 2019). A possible explanation for the observed higher resolution of the here described MLST despite the lower number of parsimony-informative sites can be the stochastic nature of mutations. The surface protein coding genes used in the MLST-s system have higher mutation rates than the house-keeping genes used by the MLST; thus, there is higher chance that mutations in the surface protein coding genes result in homoplastic alleles (Estoup et al., 2002; Urwin and Maiden, 2003).

During the comparison of the novel and previously published MLST schemes and the MLST-s system the phylogenetic trees were compiled based on typing data of the same 44 isolates with the best DNA model according to MegaX software (Kumar et al., 2018). When the same 44 isolates were analyzed the dendrogram based on the MLST system described here turned out to be more robust than of the previously published system (Tocqueville et al., 2014; Trüeb et al., 2016). The strains showed high variability based on limited number of SNPs [SNPs/concatenated sequence: 32/2304 for the previously published system (Tocqueville et al., 2014; Trüeb et al., 2016) and 73/3560 for the here described system], with both MLST systems, which might limit the robustness of the constructed phylogenetic tree. The phylogenetic tree of the MLST-s system (Clavijo et al., 2019) showed the highest robustness of the three compared MLST based methods. However, the robustness and the resolution of this system depended on the elevated number of SNPs on the surface-encoding protein gene *mltD* (SNPs/concatenated

sequence: 134/1441; SNPs/*mtlD* gene fragment: 110/537). House-keeping genes are under stabilizing selection for conservation of metabolic functions, therefore they can indicate genetic relationships more reliably than genes under positive selection, like surface protein coding genes (Urwin and Maiden, 2003); therefore the previously published and the novel MLST assays probably describe better the phylogenetic relations among the isolates compared to the MLST-s system, despite their lower robustness.

The developed MLST system is well reproducible and robust, appropriate to establish mid-term genetic relationships of the examined *M. hyorhinis* isolates. Moreover, the achieved high resolution of the developed MLST system together with detailed metadata of isolates from the same, clinically affected herd may enable the identification of the source of infection during epidemiologic investigations.

MLVA is applied to analyze tandem repeat regions, which have high mutation rates hence could detect short-term evolutional events in bacterial strains. The developed MLVA was able to differentiate isolates within farms, and within the same MLST STs. The detected high diversity of the strains is in accordance with the high genome variability of mycoplasmas (Razin, 1985). The GT/number of isolates proportion of the here described MLVA system was 92.5% (37 GT/40 isolate), higher than the MLVA system by Dos Santos et al. (2015), which was 9.69% (16 GT/165 isolate). By using more TR regions in the MLVA assay higher robustness and lower chance of homoplastic profiles can be achieved (Vergnaud and Pourcel, 2006). The established MLVA is suitable for the rapid and cost-effective fine-scale typing of the isolates, and applicable in clinical outbreaks to locate the source of *M. hyorhinis* infections.

The isolates from the same animal (MycSu32i, MycSu32s, MycSu32t) were grouped into the same MLST ST and MLVA GT, which presumes that the same *M. hyorhinis* strain caused the clinical signs in every affected tissue in this case. Two isolates (MycSu29 and MycSu101) from Bácsalmás showed the same MLVA GT, but were collected from different epidemics and

represented different STs only with the developed MLST analysis. In this case, we suppose that the stochastic nature of mutations is responsible for the detection of the same MLVA GT in two unrelated isolates (Estoup et al., 2002). These two closely related isolates were differentiated based on only one SNP detected only by the developed MLST assay, which highlights the system's suitability for the fine-scale typing of the highly variable *M. hyorhinis*, enabling the better understanding of the evolution of this pathogen.

The described MLST and MLVA assays represent convenient, well reproducible and high resolution molecular tools and revealed high variability of the examined 39 mainly Hungarian *M. hyorhinis* isolates. While the MLVA assay was suitable for the differentiation of isolates within the same MLST ST and proved to be applicable for epidemiologic studies alone, the combined MLST and MLVA method is recommended to explore both mid- and short term relations of the isolates in phylogenetic studies.

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Conflict of interest

The authors declare that they have no competing interests.

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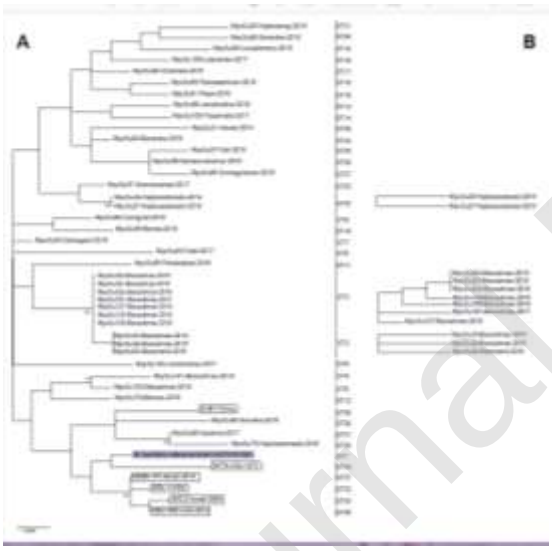
Figure captions

Figure 1

Molecular typing of 46 *Mycoplasma hyorhinis* isolates by multi-locus sequence typing (MLST) and resolution of identical sequence types by multiple-locus variable-number tandem-repeat analysis (MLVA)

A. The MLST tree was constructed by Maximum Likelihood method using gene fragments from *lepA*, *rpoB*, *rpoC*, *gltX*, *valS* and *uvrA*, with 1000 bootstraps (only bootstrap values >70% are presented). **B.** Resolution of identical STs were carried out with MLVA based on Mhr205, Mhr396, Mhr438, Mhr441, Mhr442 and Mhr444 alleles. The trees were constructed by Neighbour-Joining method.

The *M. hyorhinis* type strain is highlighted grey, the *M. hyorhinis* strains with available whole genomes are framed. Abbreviations: ST-sequence type, t-tonsilla, s-serosa, i-synovial fluid.



Tables

Table 1

Primer sequences, genome positions and sensitivity of the multi-locus sequence typing assays

Table 1

Gene	Examined region in *M. hyorhinis* type strain (NCTC 10130)	Forward primer 5'→3'	Reverse primer 5'→3'	Sensitivity[a]
lepA	92,681 - 93,174	GCT GCT ACT GAA GGT GCT TTA TTA	TGT GAA TCA ACA GGA TAA AAC CC	10^3
rpoB	71,638 - 72,295	GAT CTT CAA AAG CAG GTA ATG ATG	ATA TAG CCG ATT GAA ACA TCA TTG	10^5
rpoC	75,490 - 76,106	GTG TAC TCT ACA AAT TCT GGT GTT TCT	TGA CAA GAT AAA ATG GAA CGG AT	10^3
gltX	417,808 - 418,217	GTC AGA ATA GCT GTT TAT GAA AGT GAG	GGA CCT CTT ACT ATA TCA TTT CAA GC	10^3
uvrA	546,165 - 546,889	GTA GTT GAA CAC GAT GAA GAA ACA	CCA CCC GAT AAA GTA GTT GCT G	10^4
valS	9,426 - 10,081	ATT CAA CTC CAG GAC AAG ACA TT	TGT AAC TTT TTC TGC TTG TGG G	10^2

[a](copies/reaction)

Table 2

Primer sequences and characteristics of the tandem repeat regions of the developed multiple-locus variable-number tandem-repeat analysis

Table 2

Allele	Position in *M. hyorhinis* type strain (NCTC 10130)	Forward primer 5'→3'	Reverse primer 5'→3'
Mhr205	205,528 - 205,320	GCA GAA TCC ACT CTA GCT CAA ACT A	TTT GTA TTG TGT TCC TTT TTT TAA TCC
Mhr396	396,104 - 396,142	TAA TTT GGA TAA AAA TAC TCA ATA TGA AGT AG	AAA CTA TAC TCA ACT GTA TAT TTT GAA CCA T
Mhr438	438,283 - 438,867	CTT TAT CAA TGA ATT TTT ACA AAT GGA A	CAA ATC AAT CTG GTT CAG CAT CA
Mhr441	441,069 - 441,914	TGT GTT CTT GAT CTT TTG AGG TTT T	TCC CAA TCA CAA CAA CCA GGA

Mhr442	442,537 - 442,591	TGG TTC ATC TAC AAG CGG AGG	TGA ATC TGA TCC TGT TCC AGT CTG
Mhr444	444,088 - 444,479	ATA TTG GGT TTT TTG TTT GTA ATT TCT T	ATC AGG AAC ATC TAC AAG CGG AG

Table 3

Characteristics of the tandem repeat regions of the developed multiple-locus variable-number tandem-repeat analysis

Table 3

Allele	Repeat unit (bp)	Product length in *M. hyorhinis* type strain (NCTC 10130)	Product size[a]
Mhr205	21	183	120+21n
Mhr396	19	372	334+19n
Mhr438	39	741	178+39n
Mhr441	66	920	75+66n
Mhr442	24	151	97+24n
Mhr444	36	546	118+36n

[a]The PCR product size is the size of the VNTR flanking region plus n*repeat size, with n being the number of repeats.